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REVIEW

The stem cell and tissue engineering research in Chinese ophthalmology

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Abstract Much has been considerably developed recently in the ophthalmic research of stem cell (SC) and tissue engineering (TE). They have become closer to the clinical practice, standardized and observable. Leading edge research of SC and TE on the ocular surface reconstruction, neuroregeneration and protection, and natural animal model has become increasingly available. However, challenges remain on the way, especially on the aspects of function reconstruction and specific differentiation. This paper reviews the new developments in this area with an intention of identifying research priorities for the future.

Keywords stem cells; tissue engineering; regeneration; cell differentiation

1 Introduction

Ever since that Evans and Kanfman reported that they successfully established a mouse embryonic stem cell (ESC) line in 1981 [1], researches about ESCs were overwhelmingly worldwide. A series of ESC lines from various species were established by investigators domestic or abroad in the following 15 years. Until 1998, a milestone of stem cell research was the successful establishment of human ESC line by Thomson and Gearhart [2]. This outstanding work indicated the beginning of a new era—stem cell and regenerative medicine. Stem cell research was greatly promoted by the Chinese government and scientific grants, such as National Basic Research Program of China (973 project) and Hi-tech Research and Development Program of China (863 project). Chinese scientists seized the tide and devoted themselves into the studies of stem cells frontiers and got exciting results. In 2002, the first Chinese ESC line was established by He et al. It is well known that stem cell is a population of undifferentiated cells with the properties of self-regeneration, high proliferate ability, and multiple differentiation potentials. It can be divided into two categories according to its properties: ESC that can be induced to differentiate all kinds of cells in the body, and adult stem cell (ASC) which has finite differentiation ability, such as bone marrow stem cell, neural stem cell, retinal stem cell and limbal stem cell etc. Stem cell plays a very important role in the regeneration and reparation of ocular tissues [3]. Stem cell deficiency often leads to failure or delay of tissue recovery.

Rapid advancement of stem cell research and engineering techniques greatly promotes a new discipline—tissue engineering (TE), which includes four contents: seed cells; biological scaffold; extra-cellular niche for cells' propagation and differentiation; reconstruction techniques and transplantation *in vivo*. Proliferative and undifferentiated stem cells were inoculated into the biological scaffolds and cultured in medium with extracellular matrix and cytokines added. And then, the cell-scaffold complex was reconstructed in three dimensional culture method using engineering techniques, finally transplanted into injured tissues. Combination of stem cell with TE will bring us powerful tools to protect and repair injured tissues. The application of stem cell and TE in ophthalmology field provide an alternative branch for the regenerative medicine [4,5].

The big challenges in the development of Chinese ophthalmology are composed by the following questions: inflammation and immune response after corneal transplantation; protection and regeneration of visual function; establishment of natural animal models of ocular diseases; screening and function analysis of specific candidate genes etc. The applications of stem cell and TE in ophthalmology enable us to explore and form regenerative medicine.

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2 Applications of stem cell and TE in ophthalmology [6]

2.1 Ocular surface reconstruction

2.1.1 Limbal stem cell transplantation

Autologous limbal stem cell transplantation is performed to protect corneal ulcer, improve visual function and reduce the recurrence rate of pterygium. Furthermore, it is necessary to perform allogeneous corneal epithelium transplantation on patients with severe ocular surface disorders [7,8].

2.1.2 Establishment of human corneal epithelial cell line

The limited lifespan and quick differentiation of primary limbal stem cells greatly hindered the progress studies of corneal epithelial cells. Repeated isolation of cells from corneo-limbal tissue and time consuming primary culture were required. Therefore, an immortalized permanent cell line would facilitate studies of proliferation, differentiation and regeneration of corneal epithelium. Currently, we successfully established a corneal epithelial cell line spontaneously derived from human limbal cells which showed similar corneal epithelial cell phenotype with high proliferative capability, without tumorigenic features. It will be useful for the studies of corneal epithelial biology and the reconstructive corneal TE[1].

2.1.3 Differentiation of ESC into ocular surface epithelium

With Transwell culture system, murine ESCs were cocultured with limbal or conjunctival epithelial cells to induce differentiation [9,10]. Moreover, deepithelialized superficial corneoscleral slices were used as scaffolds to promote ESCs' differentiation into corneal epithelial cells. The results indicated that differentiated cells showed corneal epithelial cell phenotype with the expression of specific markers CK3, P63 etc [11]. In addition, allograft transplantation with cultured ESCs on amniotic membrane restored the ocular surface of severe chemically burned rabbits [12]. Therefore, ESCs could act as potential seed cells for reconstruction of ocular surface and corneal TE in the future.

2.1.4 Roles of bone marrow mesenchymal stem cells (BMSCs) in ocular surface restoration

Bone marrow mesenchymal stem cells can be readily isolated and expanded in culture and have multi-differentiation potentials. Accordingly, BMSCs have been attractive cellular tools in many clinical applications, including regenerative medicine, immune modulation and TE. In addition, the immunosuppressive properties of BMSCs make autologous cell therapy possible. In our study, we mainly focused on whether BMSCs could differentiate into corneal epithelial cells when placed in appropriate microenvironments *in vitro*. CM-DiI labeled BMSCs were cultured by Transwell culture system with Dulbecco minimum essential medium (DMEM) supplemented with EGF, TGF-β, bFGF. And then they were seeded on deepithelialized amnion membrane coated type IV collagen in a 6-well plate and co-cultured with corneal epithelial cells in the upper insert. The results showed that a series of protein markers shifted to corneal epithelial phenotype at protein or mRNA level, including E-cadherin, CD44, Integrin β1, Pax6, proliferating cell nuclear antigen (PCNA), CK12 and CK19. Now, we are carrying on further investigations regarding *in vivo* transplantation of BMSCs, and observing their phenotype, differentiation and function in rhesus monkey. *In vivo* transplantation of human MSCs to chemically burned rat cornea showed satisfying results. Interestingly, the therapeutic effect of the transplantation may be associated with the inhibition of inflammation and angiogenesis after transplantation of MSCs rather than the epithelial differentiation from MSCs [13]. Moreover, the differentiation potential of BMSCs towards conjunctival epithelium was also proved by coculturing BMSCs with conjunctival epithelial cells. And transplantation of BMSCs seeded on amniotic membrane to the ocular surface of animal model successfully restored the corneal and conjunctival surface [14].

2.1.5 Transdifferentiation of epidermal skin stem cell

In order to reduce and prevent the allograft rejection after keratoplasty, intensive and prolonged postoperative immunosuppresive therapy is necessary. Such drawbacks led us to investigate whether the ocular surface could be reconstructed by autologous epidermal skin stem cells. Our results demonstrated that skin stem cells expanded *ex vivo* were effective in restoring a normal corneal surface in the rabbit model of total limbal stem cells deficiency [15]. Recently, the experiments on monkey indicated that Rhesus putative epidermal stem cells could transdifferentiate into corneal and conjunctival epithelium-like cells and restore the ocular surface by expressing the epithelial phenotype when induced in different culture methods. In addition, the corneas post transplantation showed a smooth and clear reconstructed surface with minimal vascularization [16].

2.1.6 TE cornea

Severe shortage of donor corneas for keratoplasty greatly hindered the treatment of ocular diseases. Hu et al reported that polyglycolic acid scaffold bearing an adherent corneal stromal cell insert could be integrated into the ultrastructure of rabbit corneal stroma without compromising tissue transparency [17]. Based on the experiences that poly-lactic-co-glycolic acid was not an ideal scaffold for its translucence,

[1] Liu J B, Wang Z C, Song G. et al. Establishment of a corneal epithelial cell line spontaneously derived from human limbal cells. Exp Eye Res (under review)

topical inflammation and vascularization caused by acid degradation products [18], we tried to reconstruct TE cornea by inoculating seed cells onto the lamellar corneal stroma and cocultured with Minucell techniques. Compact and stratified corneal epithelium featured with CK3 and PCNA, vimentin expression was observed after culturing for two weeks. Our results demonstrated that dynamic culture systems had more advantages for reconstructing lamellar TE cornea than stationary culture systems on stable control of culture condition and formation of tight connection etc.

2.2 Neuroprotection and regeneration

Application of stem cell in the therapy of neuropathic diseases such as glaucoma was a hallmark of a new era—regenerative medicine. Researchers focused on regeneration of ganglion cells lost and protection of visual function, and got some valuable results. (1) Sub-retinal cavity transplantation cell tracing technique minimized the trauma of transplantation and ensured tracing implanted cells. (2) Differentiation of ESCs and BMSCs into neuron and retina-like tissue *in vivo* and *in vitro*. By injection undifferentiated ESCs into eyes of nude mice, we observed that the morphology and alignment of some differentiated cells were similar to those of the retina of nude mice. The cells were highly positive in NSE staining [19]. (3) Isolation and purification of retinal stem cells: embryonic bodies labeled with green fluorescence protein induced by retinal Muller cells and retinoic acid were selected in neural stem cells defined serum-free medium. Cells survived were identified and showed retinal stem cell phenotype. Further induction led to the expression of a series of retinal neural cells markers, such as Nestin, S100, GFAP, GAP43, Synaptophysin, Thy1.1, and MAP2 etc. Investigations about the function of differentiated cells should be performed to get the functional restoration of cell transplantation [20]. (4) Autologous hippocampus derived neural stem cells were successfully induced into retinal lineage cells and would provide an alternative seed cells for regenerative medicine of ophthalmology [21]. (5) Isolation and culture of tumor stem cells in human retinoblastomas (RB): RB cells isolated from RB tumors were cultured *in vitro* and identified the proliferating and differentiative properties. The results showed that RB cells could form proliferative neurospheres which could self-renew and express retinal progenitor cell related genes, and when they were transferred to induction medium, they could differentiate into neurons and glia. Our findings demonstrated that there were subsets of tumor stem cells resembling retinal progenitor cells in human RB which could act as seed cells in TE and a target of cell in gene therapy [22].

2.3 Gene targeting and establishment of natural animal model

Stem cell provides us a unique tool for studies of gene function and mechanism of ocular diseases. Screening mutant genes causing ocular diseases should be confirmed by investigations of gene function in animal models. Recently, we performed genome-wide scanning and linkage analysis to screen the candidate genes in a large Chinese pedigree (GZ.1 family) with familial open-angle glaucoma. The Lod scores mapped the candidate gene in GLC1A locus of chromosome 1. Mutation analysis showed that the Pro370Leu mutation of Myoc/TIGR gene co-segregated with all affected individuals. In transfected human trabecular cells, we found that mutant Myoc/TIGR protein accumulated as aggresome-structures in trabecular cells and led to cytotoxicity. To elucidate the pathogenic process from gene to organ, natural open-angle glaucoma models will be constructed using stem cells and spatiotemporal gene targeting technique [23,24]. Natural animal models mimicking the process of diseases help us elucidate the detailed symptoms and signs, and could direct the diagnosis and treatment of such diseases. Current animal models were scarcely naturally derived. Furthermore, most of them were from rodent species. The gene regulation pattern, biological features and functional characteristics of rodent differ greatly from that of primates and human. Therefore, the ideal animal model should be based on the combination of stem cell and gene targeting techniques to establish natural primates ocular diseases models. In our preliminary study, chimera animal model was constructed and applied for the investigations of transdifferentiation of epidermal skin stem cell. As we know, chimera animal model is very useful not only in the field of gene function and pharmacology, but also in the field of stem cell researches [25–27]. It will be a good model to investigate how cells injected into a blastocyst migrate and differentiate. If they really contribute to the development of different organs, it will help us to focus on the molecular mechanisms of such phenomena. Purified rhesus epidermal skin stem cells were injected into the blastocytes of Balb/c mice. Up to now, we have got three chimera mice. Further characterization of skin stem cell location, phenotype and plasticity of implanted rhesus skin stem cells are being carried out.

3 Challenges and future of stem cell and TE

The research of stem cell and TE brought us such a wonderful view of regenerative medicine. However, we must face many challenges and deal with them properly. (1) Precise modulation of proliferation and differentiation: only when we know the exact regulation mechanism of this differentiation system, can we control the process of induction and obtain specific cells or tissues we want. (2) Isolation and purification of stem cells: up to now, we still don't know the specific markers to identify and purify stem cells from various cell populations. Therefore, investigations focusing on monoclonal antibodies recognizing special markers of stem cells would promote greatly the stem cell research. (3) Tumorigenesis and immunogenesis of stem cells: pluripotent stem cells are usually tumorigeneous when they were implanted *in vivo*. Thus, more and more researchers would like to use tissue specific

autologous stem cells (ASCs) for cell therapy, since they can supplement the injured tissue with low tumorigeneous probability. Meanwhile, ASCs transplantation and individual tissue reparation design were performed to avoid the immune allograft rejection. (4) Function analysis of stem cell transplanted *in vivo*: Not only structure restoration but also functional recovery have been accomplished, would the therapy be meaningful. It is especially important for studies of regenerative medicine to evaluate function of integrated stem cells in damaged animal models. Therefore, recently, patch clamp technique has been used to record electrophysiological features of neurons and corneas cells [28,29] (5) Ethnics problems: researches of stem cell and TE are related to many ethnical problems. Laws and regulations should be established to control and monitor these researches. (6) It will be given special emphasis on the tissue construction in large mammalian animals in order to establish a solid scientific basis for clinical application of engineered tissues [30].

In summary, this short paper offers a new insight into the research field of stem cell and TE in Chinese ophthalmology. How beautiful prospect it shows, stem cell and TE still have a long way to go. Moreover, it is very important to translate the findings of basic research to clinical practice. Fortunately, eye is a special organ suitable for such translation due to unique features, which is accessible, visible and measurable. Stem cell and TE will have more and more applications in ophthalmology, and will promote the development of ophthalmology greatly [31].

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